Saskatchewan Agriculture, Food and Rural Revitalization

ADF

AGRICULTURE DEVELOPMENT FUND

FINAL REPORT

20020153

INDUCTION OF PROTECTIVE MUCOSAL IMMUNITY AGAINST COLIBACILLOSIS IN POST-WEANING PIGS

Funded by: Agriculture Development Fund

March 2005

Prepared by: VIDO

106-01273/

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Feb. 2005

INDUCTION OF PROTECTIVE MUCOSAL IMMUNITY AGAINST COLIBACILLOSIS IN POST-WEANING PIGS

Project 20020153

Volker Gerdts* and Philip Willson

Vaccine and Infectious Disease Organization
University of Saskatchewan
120 Veterinary Rd.
S7N 5E3 Saskatoon

***Phone: (306)-966 1513**
Fax: (306)-966 7478
Email: gerdts@sask.usask.ca

I. Abstract

Post-weaning colibacillosis (PWC) is an economically important disease of swine caused by infection with *Escherichia coli*. The disease is typically found one to two weeks post weaning. The disease outcome is determined by the presence of the F4 (fimbriae antigen 4, or K88) receptor (F4R) on the surface of the intestinal epithelium. Only F4R$^+$ pigs are susceptible to the disease and display clinical symptoms. Experimental vaccines based on live-attenuated bacteria or purified F4-protein demonstrated that intestinal immunity is crucial for protection. We developed new mucosal vaccine formulations and analyzed the induction of local immunity against PWC.

II. Executive summary

II.1. Background: PWC is caused by F4-expressing enterotoxogenic *Escherichia coli* (ETEC). The disease is responsible for substantial economic losses to producers worldwide. Typically occurring within one to three weeks after weaning this enteric disease is characterized by high morbidity and moderate mortality. Symptoms include diarrhea, fever, and losses in weight gain. Immunoprophylaxis of post weaning colibacillosis (PWC) still represents a major challenge as there are currently no effective vaccines commercially available. Only vaccines that induce immunity at the mucosal surfaces of the small intestine are protective against infection. **Thus, the goal of the present project was to develop mucosal vaccines to induce protective immunity at the intestinal mucosal surfaces.**

II.2. The role of the F4-receptor: The F4-receptor (F4R) comprises a group of receptor molecules that are found on the surfaces of the intestinal epithelium and that bind the F4 antigen with varying affinity (Fig. 1). Only F4R+ pigs are susceptible to infection and develop disease. In addition, it was proposed by Cox et al. (2000) that only F4R+ pigs respond to vaccination, whereas vaccination of F4R- pigs fails to induce protective immunity against challenge infection (Van den Broeck, 1999; Cox 2002). This observation clearly challenged our understanding of the mucosal immune system in the intestine since F4R$^-$ animals would still possess the 'normal' M cell- and dendritic cell-mediated ways of antigen-uptake and subsequent induction of local immunity. Thus, at least theoretically, these animals should still be able to mount immune responses against the F4. It was therefore suggested by the same authors that F4R$^-$-animals develop oral tolerance instead of an immune response against the F4. However, in none of these studies was the F4 protein delivered locally, which would reflect the way of natural exposure in pigs. **Thus, an important aspect of our research was to test if local immunization using our recently developed gut-loop model would result in induction of F4-specific immune responses in F4R$^-$ animals.**

II.3. Local immunization with F4 into gut-loops: We established the protocols to purify the F4 antigen, to determine the presence of the F4R in the intestine and to determine the immune response based on the presence of F4-specific antibody-secreting

cells in immunized gut-loops. The gut-loop model was recently developed in large animals and is based on the surgical creation of independent loops. We used this model to analyze the local immune response against the F4 following local immunization (Fig. 5-7). We performed three independent sets of experiments (total of 14 animals, see table 1) to determine the i.) dose of F4 for immunization, and the ii.) time interval between immunization and collection of the loops. Doses between 50-500 µg/loop of F4 were used for intestinal immunization. **Interestingly, our results demonstrated that only F4 R+ animals were able to mount a significant immune response against the F4.** However, the level of expression of the F4R greatly varied among analyzed animals and consequently the observed immune responses varied greatly in their magnitude. Our data confirmed a strong correlation between the F4 expression level and the magnitude of the immune response in the small intestine (Danabassis, *manuscript in preparation*). In contrast, F4R-pigs did not respond to vaccination indicating that the presence of the F4R is essential for the induction of local immunity in the intestine. **We therefore hypothesized that the addition of potent adjuvants could overcome the induction of tolerance in pigs and induce an immune response even in F4R- pigs.** We tested two potent mucosal adjuvants to i.) enhance the local immune response and to ii.) induce local immunity in F4R- pigs. We used CpG oligodeoxynucleotides (CpG ODN) and the porcine beta-defensin 1 (pBD-1) for adjuvanting the F4.

II.4. CpG ODNs as mucosal adjuvants: Bacterial DNA is a pathogen associated molecular pattern (PAMP) recognized by vertebrates and has potential as adjuvant or immunotherapeutic reagent (28). The stimulatory effect is due to the presence of unmethylated CpG dinucleotides (CpG motifs) within the context of certain flanking bases (13, 76). Synthetic ODNs that contain CpG motifs similar to those in bacterial DNA also have immunostimulatory capacities and overall promote the production of Th1 and pro-inflammatory cytokines and induce the activation/maturation of antigen-presenting cells (APCs). CpG ODNs have been successfully described as potent mucosal adjuvants in a variety of species. VIDO has gained significant experience within the last years using CpG ODN as vaccine adjuvant and stand alone innate immune treatments. In fact, VIDO was one of the first groups in the world to report the potential of CpG ODN for livestock vaccines. We used the CpG ODN 2007 in our experiments. This class B ODN has been demonstrated to be a strong adjuvant in pigs. The F4 protein and the CpG ODN were mixed shortly before injection into the gut-loops.

In total, six animals were immunized with F4 (125 µg/loop) and CpG ODN. In each animal three loops were immunized with F4 (125 µg/loop), three loops were immunized with F4 (125 µg/loop) and CpG ODN (500 µg/loop), and two loops were treated with saline (PBSA) control. Loops were collected after 21 days and the number of F4-specific IgA and IgG-antibody secreting cells determined. Surprisingly, the addition of CpG did not result in an enhancement of the magnitude of the immune response in immunized loops (Fig. 7). Furthermore, lymphoproliferatives responses were not enhanced by adjuvanting with CpG. **Thus, our results indicate that the addition of CpG ODN as a mucosal adjuvant had little or no effect on the immune response in the small intestine of immunized pigs.**

II.5. Antimicrobial peptides as mucosal adjuvants: Since the addition of CpG ODN had little or no effect on the magnitude of the immune response, we tested other potential adjuvants for mucosal vaccines against *E.coli* infections. We tested the potency of the porcine beta-defensin 1 (pBD-1) for enhancing the specific immune response against *E. coli*. A total of seven animals were immunized with the F4 or F4 in combination with pBD-1. Three loops each were immunized with F4 (125 µg/loop) or F4 (125 µg/loop) and pBD-1 (250 µg/loop). Two loops were treated with saline as control. Surprisingly, combination with pBD-1 did not result in an enhancement of the local immune response, as shown in Figure 7. In contrast, addition of pBD-1 seemed to decrease the number of F4-specific IgA- and IgG-antibody secreting cells. **Thus, neither CpG ODN nor pBD-1 augmented the local immune response**.

II.6. Summary: Taken together, we have developed a potential mucosal vaccine against post-weaning colibacillosis in pigs. The vaccine was tested in surgically created loops and was found to be effective for inducing local immunity in the small intestine. Optimal doses were found to be between 50-500 µg/loop of F4. Interestingly, the magnitude of the immune response correlated with the expression levels of the F4R. In order to reduce the amount of antigen needed for vaccination and possibly induce protective immunity in F4R- animals, we tested two novel adjuvants in combination with the F4. Surprisingly, addition of either of the two adjuvants resulted in an enhancement of the vaccine efficacy. Thus, we recommend analyzing other potential vaccine strategies such as the use of viral vectors for inducing local immunity in the small bowel. We are currently investigating the potential of combining DNA vaccines with purified proteins (prime-boost strategy) to enhance the immune response against *E.coli* infections in pigs. However, our results also suggested a novel strategy for improving oral vaccines by targeting to antigen-specific receptors (F4R, see below).

II.7. Targeting oral vaccines to the intestinal mucosa (immunization into interspaces, loops without Peyer's patches): Since our results suggested that the presence of the F4R is essential for induction of local immunity, we analyzed the possibility of targeting vaccines to the F4R to induce/enhance the local immune response (Fig. 8). We received additional funding for this project from the Canadian Network on Bacterial Pathogens of Swine and the Saskatchewan Health Research Foundation (Post-Doctoral Fellowship for Dr. M. Salles). The rationale for this approach is to target oral vaccines to the F4R by fusing the vaccine antigen to the F4-gene. Binding via the F4 will mediate the uptake of the vaccine antigen, and thus result in induction against the vaccine antigen. We will focus on rotavirus vaccines since rotavirus infections are responsible for a number of important diseases in humans and livestock.

We have started by immunizing 6 animals with F4 (250 µg/loop) into interspaces, three interspaces immunized with F4 and three interspaces treated with saline. **Interestingly, we found strong immune responses in immunized interspaces in F4R+ animals, but no immune response in F4R- animals.** Again, the magnitude of the immune response

correlated with the expression level of the F4R. Thus, our results suggest that the binding of the F4 to the F4R is necessary for the induction of a local immune response in the gut. Since interspaces lack organized lymphoid tissue (Peyer's patches) and specialized cells for antigen-uptake (M cells) one present hypothesis is that dendritic cells take up the antigen and induce local immunity within the interspaces. **This has great importance for the development of novel oral vaccines since this strategy would greatly enhance the efficacy of oral vaccines.** We are currently creating a fusion protein consisting of the F4 and the rotavirus VP6 to test our hypothesis in pigs.

III. Technical report

III.1. Purification of the F4-antigen and the impact on its immunogenicity: We have established two ways of purifying the F4 antigen. The first method is based on shearing the assembled F4 fimbrae off the bacteria followed by washing and dialyzing against saline ('non-denatured'; Fig 3). The second method allows the isolation of a more purified protein and is based on subsequent SDS-polyacrylamide gel purification, and electroelution ('pure'). Specificity of the F4-protein purified by either method was confirmed by western blot using the polyclonal rabbit sera we produced, or sera provided by J. Fairbrother, University of Montreal. However, the 'non-denatured' F4 was more immunogenic than the 'pure' F4 and thus was used for all immunizations. The 'pure' F4 was used as antigen in immune assays, such as immunohistochemistry and ELISA, where a more specific antigen is needed to reduce the chance of unspecific binding.

III.2. Bacterial adhesion assay: The presence of the porcine receptor for F4 (F4R) is genetically determined and can be verified using bacterial adhesion assays (Fig. 1 and 2). We developed this assay in our laboratory based on the isolation of villi from the intestine at the time of surgery and again at the collection of the loops. However, this assay is very sensitive to the quality of the villi and works only with freshly isolated material. Thus, we developed an alternative assay using immunohistochemistry. This assay is based on biotinylation of the F4-protein, which then can be detected by peroxidase staining. The amount of bound F4-protein correlates to the number of receptors expressed on the surface of epithelial cells (Salles et al., 2004). Both assays were used in parallel for each piglets for quantifying the level of F4R-expression.

III.3. Pig challenge model: In order to analyze the efficacy of experimental vaccines it is important to establish a challenge model in specific pathogen-free weaned pigs. However, as shown by other researchers (Van den Broeck et al. 1999; Francis and Willgohs 1991; Madec et al. 2000) the development of a PWC-challenge model is complicated by the fact that a number of factors play a role in the onset of the disease. We performed two challenge experiments. We used the F4-strain obtained from Drs. C. Gyles (Guelph) and J. Fairbrother (St. Hyacinthe) in both experiments. Bacteria were grown on Brain-Heart agar plates and washed in saline before inoculation. All pigs were pretreated three days prior to challenge with antibiotics (Nuflor™, Schering-Plough) given orally to experimentally disturb intestinal flora. Prior to inoculation the piglets received about 60 ml bicarbonate buffer to neutralize the acidic environment in the stomach. Each animal was inoculated with 5 ml of bacterial suspension containing 5 x 10^{10} cfu of ETEC F4.

Animals were monitored daily for clinical symptoms, diarrhea, and weight loss. Additionally, fecal swabs were taken daily to determine the amount of bacterial shedding on blood-agar plates. Strains were identified by dot blot analysis using F4-specific polyclonal antibodies. The mortality was > 60% in our model with bacterial shedding for > 6 days in all infected animals. None of the control animals shed F4$^+$ *E.coli* during the

experiment. Thus, we developed the challenge model in post-weaning pigs for testing disease protection in F4-immunized pigs.

III.3. Immunization with the F4 into surgically created gut-loops: A number of gut-loop experiments were performed to determine the local immune response in the small intestine against *E.coli* (Fig 4):

a.) Dose response: 500 µg of F4 was used initially for immunization into each gut-loop (Table 1, Expt. IV), Depending on the expression levels of the F4R moderate to strong immune responses were detected in each immunized loop. However, to analyze the effect of mucosal adjuvants, we needed to determine the lowest dose that consistently resulted in a detectable immune response. To this end 50, 250 or 500 µg of F4/ loop were used for immunization into two loops each in six animals (Table 1, Expt. V.). F4-specific immune responses were found in all F4R+ pigs (Fig. 5, 6). The magnitude of the detected immune response seemed to correlate to the concentration of the F4R expressed.

b.) Testing CpG DNA as mucosal adjuvant:
Since 50 µg of F4 resulted in a detectable immune response in all immunized loops, we decided to use this dose for analyzing the effect of CpG DNA or defensin as mucosal adjuvants. In a total of six piglets gut-loops were surgically created and three loops were immunized with F4 alone, and three loops were immunized with F4 plus 500 µg of CpG (Expt. VI). Surprisingly, addition of CpG did not enhance the local immune response against the F4 (Fig. 7).

c.) Testing pBD-1 as mucosal adjuvant: During the time period of this grant, our lab started to work with antimicrobial peptides which are important components of the innate immune system (research was funded by the Canadian Bacterial Disease Network, Young Investigator Award). We demonstrated that the pBD-1 is responsible for protecting piglets against an experimental infection with *B. pertussis*, the causative agent of whooping cough in humans (Elahi et al., 2004, Elahi et al, manuscript in presentation). We also demonstrated that the pBD-1 has antimicrobial activity against a broad range of bacteria including *Actinobacillus pleuropneumiae* and *Streptococcus suis*. Furthermore, the pBD-1 displays strong immunomodulatory activity *in vitro* and induces the upregulation of IL-4, IL-12 and IFN-γ in various immune cells (Lymph nodes, Peyers patch and blood). Evidence from the literature confirms that AMPs, in particular defensins are powerful immunomodulators which can be used as vaccine adjuvants (reviewed in Ganz, 2003; Zasloff 2002). We therefore decided to investigate if the pBD-1 can be used to enhance the immune response against *E. coli*.

First, we analyzed the inhibitory activity of the pBD-1 *in vitro* by co-culturing *E. coli* with different concentrations of pBD-1. As shown in Fig. 10, the pBD-1 reduced the growth of the F4 expressing *E.coli* (ETEC F4) by more than 1,000,000 fold within six hours. This clearly demonstrates that the defensin on its own has antimicrobial activity against the ETEC F4. Furthermore, when co-cultured with Peyer's patch or lymph node cells the pBD-1 induced a significant upregulation of proinflammatory cytokines and

interleukins. This is important for recruiting immune cells such as dendritic cells and macrophages to the site of immunization and furthermore for providing the local microenvironment for inducing an immune response.

A total of seven animals were immunized with the F4 or F4 in combination with pBD-1. Three loops each were immunized with F4 (125 µg/loop) or F4 (125 µg/loop) and pBD-1 (250 µg/loop). Two loops were treated with saline as control. Surprisingly, combination with pBD-1 did not result in an enhancement of the local immune response, as shown in Figure 7. In contrast, addition of pBD-1 seemed to decrease the number of F4-specific IgA- and IgG-antibody secreting cells. No effect was observed on lymphoproliferative responses against *E.coli*. However, the pBD-1 was simply added to the vaccine prior to inoculation into the gut-loop. To allow close proximity between antigen and adjuvant we created a fusion protein (F4-pBD-1) and expressed this fusion protein in baculovirus.

III.4. Creation of an F4-pBD-1-fusion protein: In addition to simply adding the pBD-1 to the vaccine formulation, we are currently creating a fusion protein based on the F4 and the pBD-1. The idea is that the pBD-1 part of the fusion protein would attract and activate dendritic cells which then could efficiently present the F4 to lymphocytes thereby enhancing the immune response against the F4. A similar approach was demonstrated for the HIV gp120 antigen linked to the murine beta defensin-2. In mice, the fusion to the beta-defensin resulted in stronger humoral and cellular immune response against the gp120. To create the F4-pBD-1-fusion protein the gene encoding the F4 was amplified by PCR and fused to the gene encoding the mature peptide of pBD-1. Additionally, a linker sequence was introduced in between to enable the correct folding of both individual peptides. The fusion protein was expressed in baculovirus. The activity of both proteins will be assessed by *in vitro* growth inhibition and inhibition of binding to the F4R in bacterial adhesion assays. Once this has been determined the fusion protein will be used for immunization.

III.5. Immunization into interspaces (loops without Peyer's patch): Four pigs were immunized with each 500 µg F4/interspace (n= 4/ pig) and with saline (n= 4/ pig). Control loops (containing a Peyer's patch) were treated with saline. Loops and interspaces were collected after 21 days and cells were isolated from both Peyer's patches and gut-wall. Interestingly, local immune responses were only found in immunized interspaces but not in adjacent control interspaces (treated with saline) or control loops (Peyer's patch, treated with saline, Fig. 10). The magnitude of the immune response in each immunized interspace correlated to the level of F4R-expression. Thus, we demonstrated that the presence of the F4R is essential for inducing local immunity in the small intestine. Furthermore, in contrast to previous experiments in sheep using chicken ovalbumin (OVA) for immunization we showed that even in interspaces that lack organized lymphoid tissue primary immune responses can be induced. We speculate that dendritic cells mediate the uptake and the induction of local immunity. This could have great potential for developing novel strategies for future oral vaccines (Fig. 9).

IV. Personnel

The ADF-funding was used to pay for the salary of Ms. Rachelle Buchanan, Research Technician Level III. Additonally, through matching funding from SHRF and CNBPS we were able to employ Dr. Monica Salles (Post-doctoral fellow) and Mr. Michael Danabassis, Master's graduate student, in this project.

Matching funding: The Canadian Research Network on Bacterial Diseases of Swine funded our project in their New Innovative Research Competition Oct. 2003. The aim of the project is to determine the role of the F4R for targeting oral vaccines to the intestinal mucosa of pigs. The project was funded with Can $ 12,000. It was suggested to submit a full-proposal at a later time-point.

In addition, we received a Post-Doctoral Fellowship for Dr. Monica Salles (DVM, PhD) from the Saskatchewan Health Research Foundation (SHRF) for two years (Can$ 90,000; Aug. 2004-2006). Dr. Salles currently investigates the role of the F4R for targeting oral vaccines to the intestinal mucosa.

Collaborations: We intensified our collaborations with researchers from the Canadian Network for Bacterial Pathogens of Swine (CNBPS; Drs. Gyles, Fairbrother, Jaques). All results are discussed with Dr. Gyles at a regular basis, and joint experiments and grant applications are planned for the future. The CNBPS has submitted a letter of intend for another five years. In this application, under the leadership of Drs. Willson and Gordon (WCVM), VIDO will become the porcine immunology/ vaccine development node of the network.

V. Equipment

No equipment was purchased.

VI. Project Developed materials

VII. Project Photos

VIII. Acknowledgements

Data was presented at:

1. Danabassis, M.S., M. Salles, J. Foley, P. Willson, L.A. Babiuk, and V. Gerdts (2005) Enhancing protective immunity against colibacillosis in post-weaning pigs. Research Day at the University of Saskatchewan, March 2005 (poster).

2. Salles, M. (2004) The *Escherichia coli* F4-Receptor for Targeting Oral Vaccines to the Intestinal Mucosa. Annual Meeting of the Canadian Research Network on Bacterial Diseases of Swine 2004, University of Guelph, June 8-10, 2004 (oral presentation and poster).

3. Gerdts, V. (2004) Understanding mucosal immunity- Development of oral vaccines against *E. coli* infections in pigs. Annual Meeting of the Canadian Research Network on Bacterial Diseases of Swine 2004, University of Guelph, June 8-10, 2004 (oral presentation).

4. Danabassis, M.S., M. Salles, J. Foley, P. Willson, L. Babiuk, and V. Gerdts. (2004) Enhancing protective immunity against colibacillosis in post-weaned pigs. Research Day at the University of Saskatchewan, March 2004 (poster).

X. CAR Data Entry

XI. References

1. Cox, E., Y. van der Stede, F. Verdonck, V. Snoek, W. van den Broeck, and B. Goddeeris. 2002. Oral immunization of pigs with fimbrial antigens of enterotoxigenic *E. coli*: an interesting model to study mucosal immune responses. Vaccine 87: 287-290

2. Elahi, S., R. Brownlie, J. Korzeniowski, R. Buchanan, B.O'Connor, M.S. Peppler, L.A. Babiuk, S.A. Halperin, S.F. Lee, and V. Gerdts. Infection with Bordetella pertussis in newborn piglets: a new model for whooping cough, *submitted to Infection and Immunity.*

3. Elahi, S. J. Korzeniowski, R. Buchanan, R. Brownlie , M.S. Peppler, L.A. Babiuk, and V. Gerdts. Expression fo the procine beta defensin-1 is essential for disease protection in a porcine model of pertussis. 12th International Congress of Immunology and 4th Annual Conference of FOCIS, July 18-23, 2004. Montreal Canada.

4. Ganz, T. 2003. Defensins: Antimicrobial peptides of innate immunity. Nat. Rev. Immunol. 3: 710-720.

5. Van den Broeck, W., E. Cox, and B.M, Goddeeris. 1999. Receptor-dependent immune responses in pigs after oral immunization with F4 fimbrae. Inf. Imm. 67: 520-526.

6. Zasloff, M., 2002. Antimirobial peptides of multicellular organisms. Nature 415: 389-396.

XII. Attachments

- Figures 1-
- Table 1

Bacterial adhesion assay (villous adhesion assay)

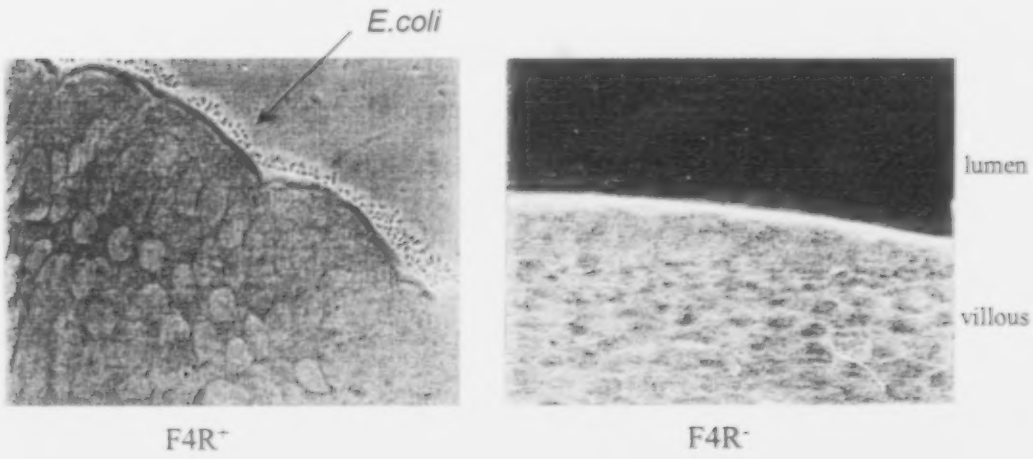


Fig. 1: Pigs that have F4R are colonized with *E.coli* (left), and consequently, can develop disease or immune protection. In contrast, pigs lacking this receptor (right) do not get sick or develop an immune response against *E. coli*.

Specificity of the villous adhesion assay

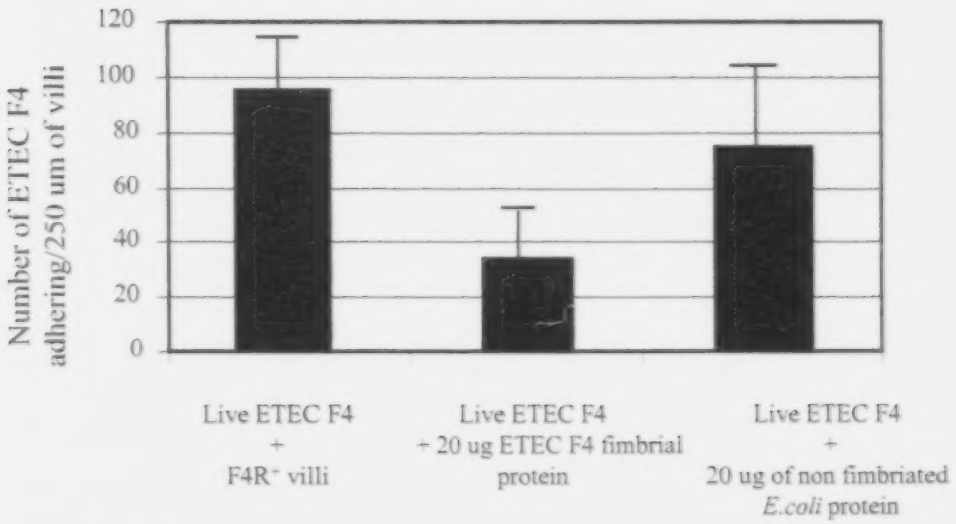


Fig. 2: Blocking adherence of *E.coli* to the F4R. To demonstrate the specifity of the assay bacteria were pre-incubated with 20 ug ETEC F4 fimbrial protein and subsequently incubated with freshly isolated villi. Adherence of the bacteria was significantly reduced after preincubation with F4.

Isolation of the F4

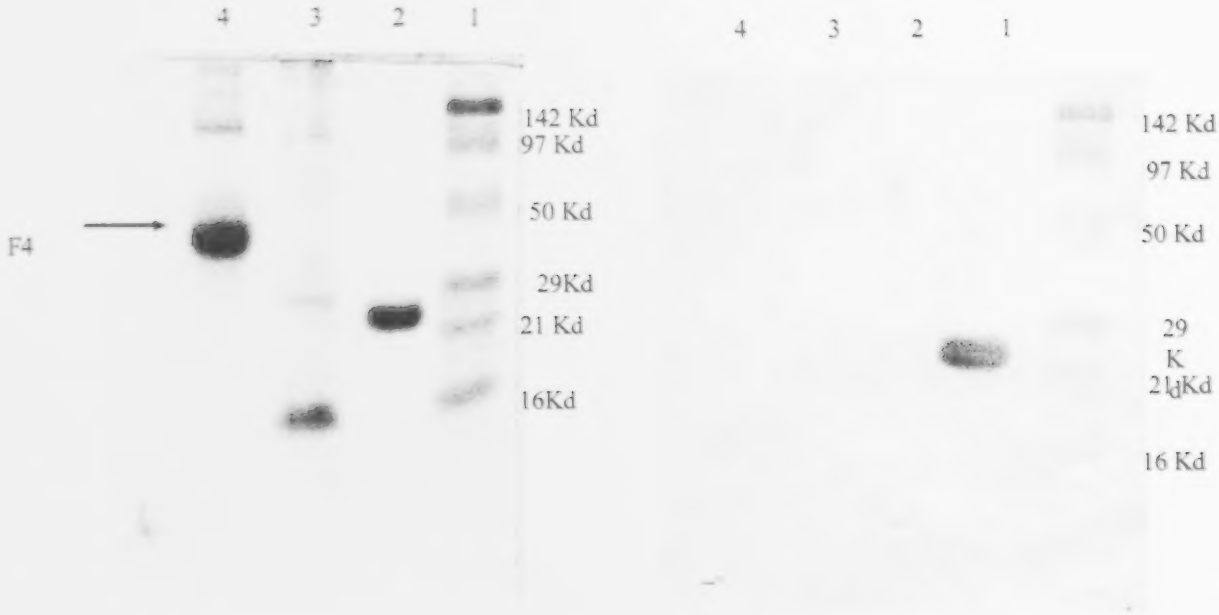


Fig 3: SDS PAGE and Western Blot were used to isolate the F4 (26KD).
Briefly, overnight cultures of ETEC bearing F4, and EL14 (a non fimbriated *E.coli* strain) were processed by centrifugation, shearing, ammonium sulphate precipitation, and overnight dialysis in dd H_20. The F4 was subsequently purified using SDS PAGE and electroelution.
5 ug of protein was loaded per well. Lane 1 = F4; lane 2 = EL14; lane 3 = ovalbumin (OVA).
A polyclonal serum derived from F4-immunized rabbits was used for detecting purified F4 in our Western Blot.

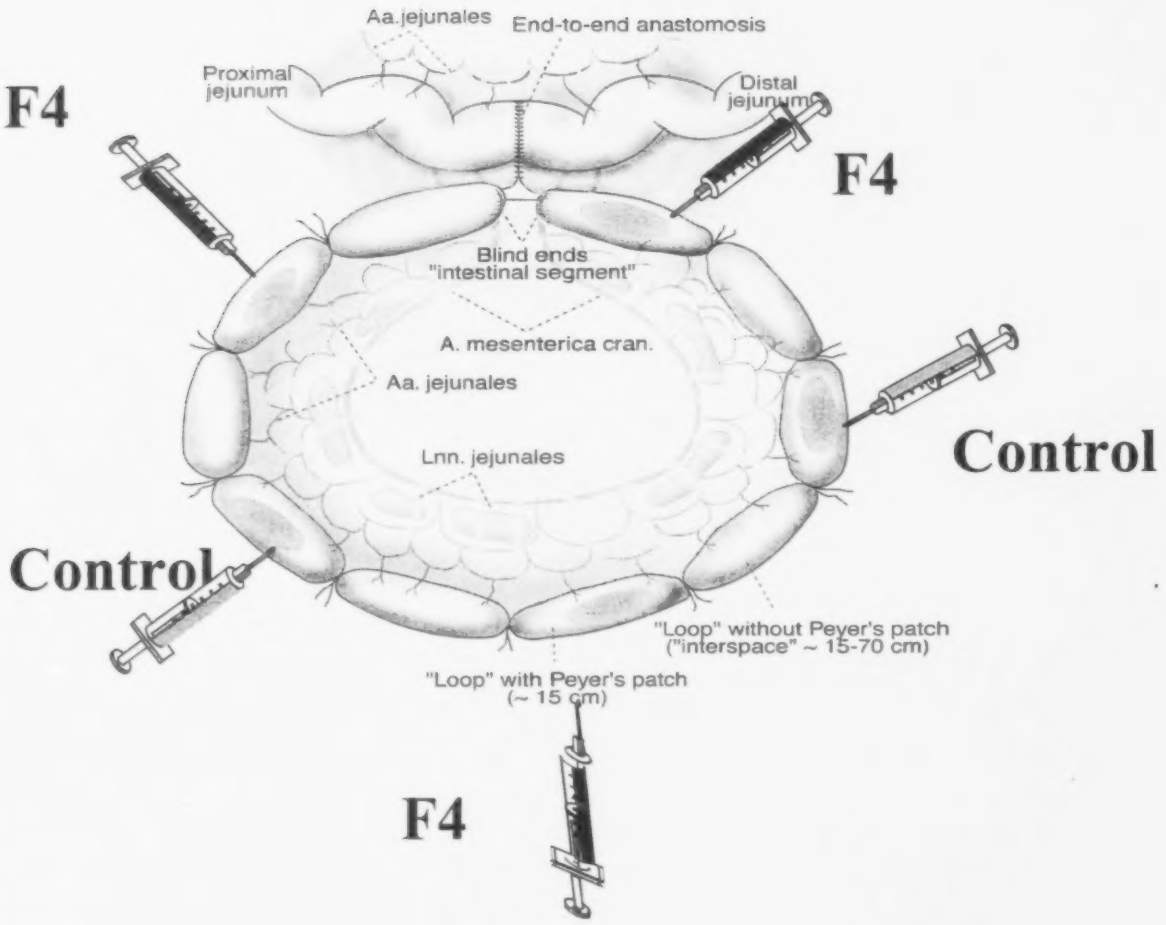
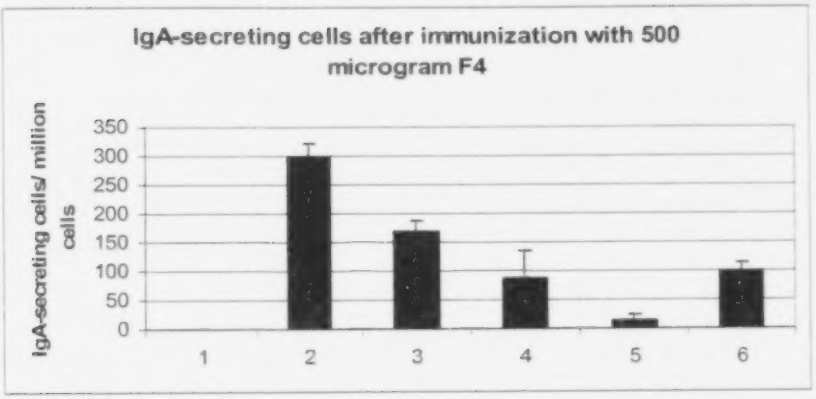
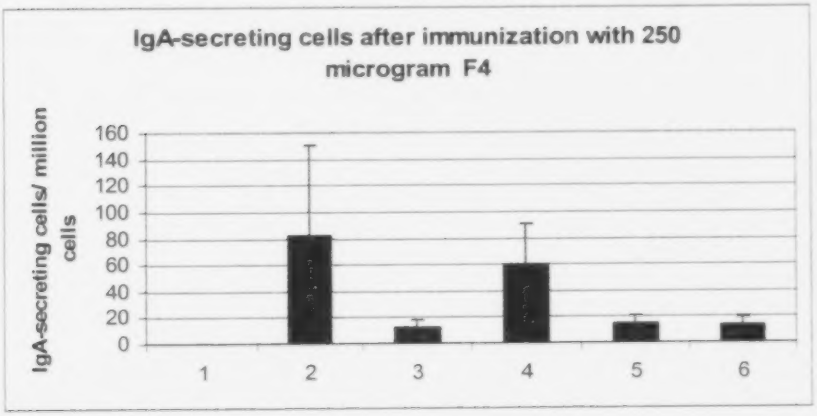


Fig.4: The gut-loop model. Intestinal loops were surgically created in four week old piglets. Each loop (containing a Peyer's patch) or interspace (lacking a Peyer's patch) were immunized With varying amounts of F4 or saline (controls). After three weeks all loops were collected and cells were isolated from Peyer's patches and interspaces and assayed in a variety of immune assays.

A.



B.



C.

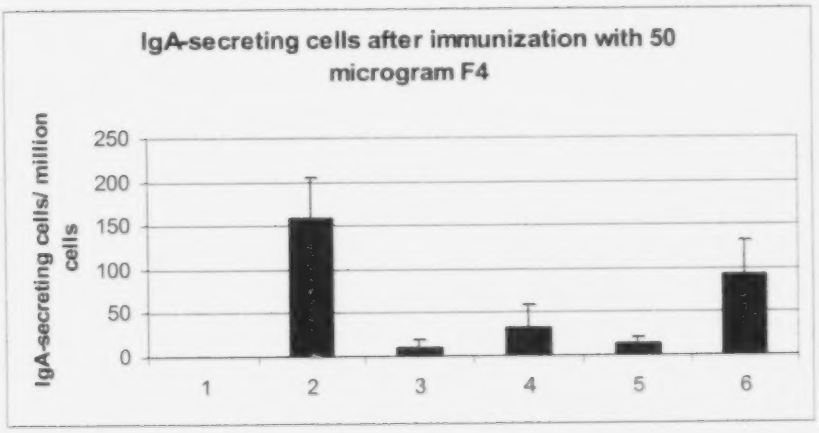


Fig 5: Six pigs were immunized with varying doses of F4 (500, 250, or 50 μg F4; A-C) into two loops each. The loops were collected after three weeks and cells were isolated from Peyers patches and gut-wall. Solid bars represent the mean (with T-bars indicating the standard deviation) of F4-specific IgA-secreting cells in Peyers patches.
Animals # 218 (2), #221 (4), and #223 (6) expressed large concentrations of F4R. Animals #220 (3), #222 (5) expressed low concentrations of F4R, and # 217 (1) was negative for the F4R.

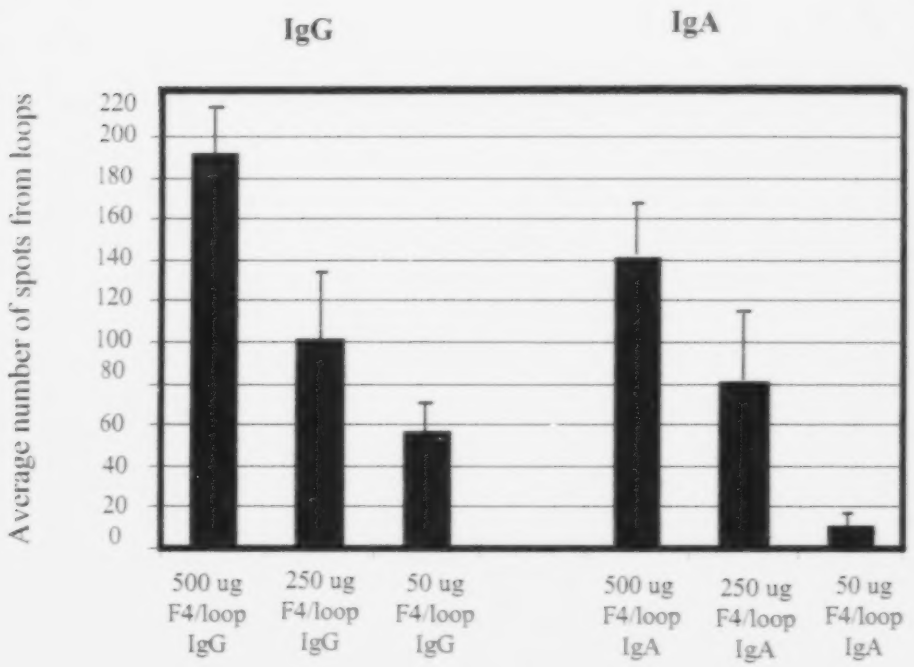
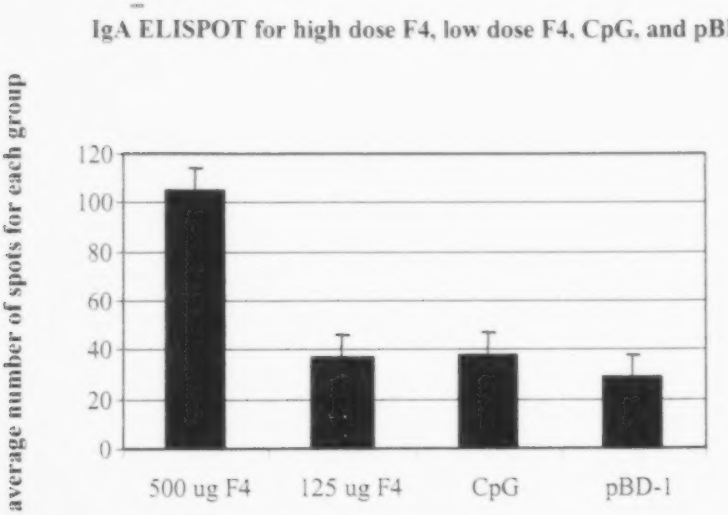


Fig.6: Varying amounts of F4 (500-, 250-, and 50 ug F4/loop) were used for immunization. The number of F4-specific IgA- and IgG antibody secreting cells were determined in a modified ELISPOT assay.



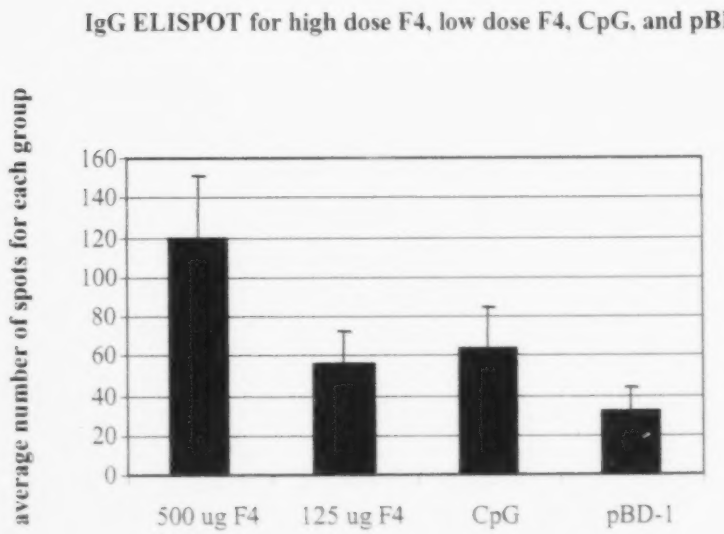


Fig.7: Varying amounts of F4 (500, 250, and 50 ug F4/loop) and combination of 125 ug F4/loop plus either 500 ug CpG/loop or 250 ug pBD-1/loop were used for immunization. The number of F4-specific IgA- and IgG antibody secreting cells were determined in a modified ELISPOT assay.

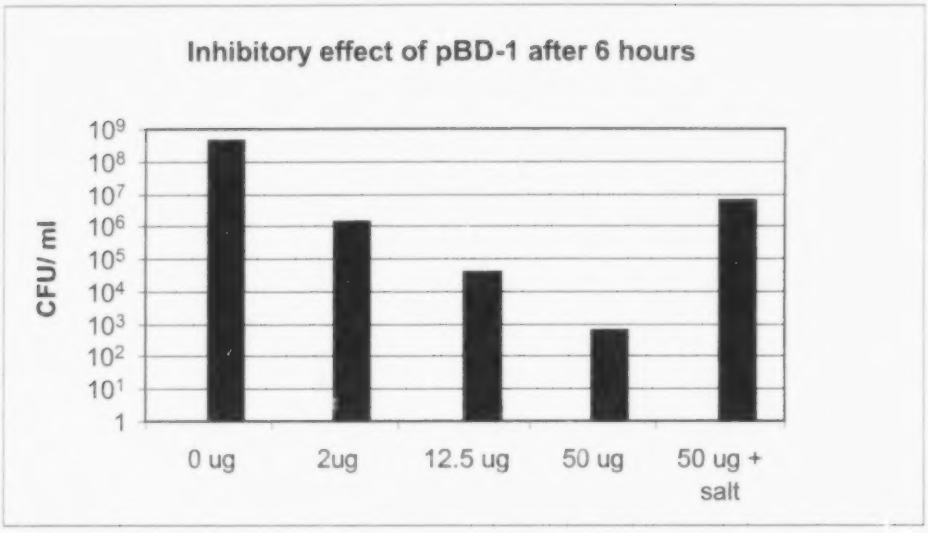


Fig. 8: 2 x 10^3 CFU of ETEC F4 were co-cultured with varying amounts of pBD-1 in 200 µl of media. After 6 hours the supernatants were plated onto LB-agar plates to assess the amount of bacteria. Solid bars represent the amount of *E.coli* after 6 hr treatment with pBD-1.
High concentrations of salt inhibit the effect of antimicrobial peptides. To prove that the observed inhibitory effect was related to the presence of the pBD-1 high concentrations of salt were added as internal control (last column).

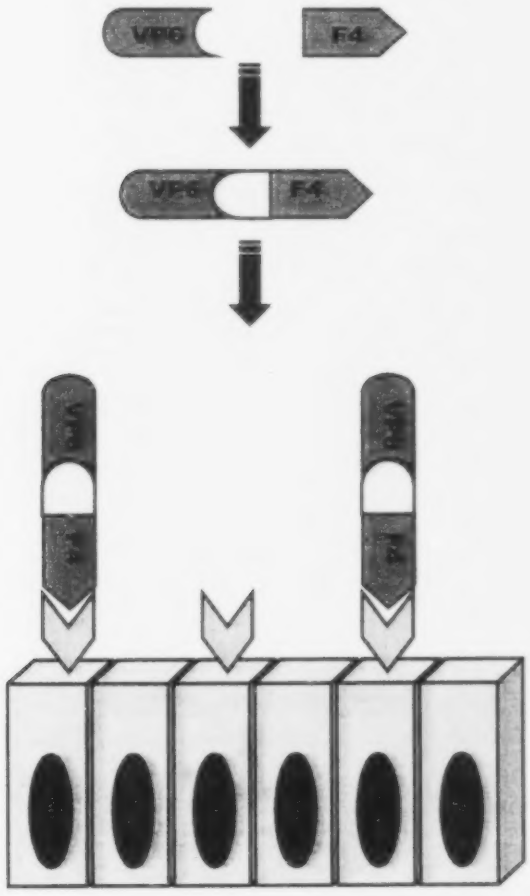
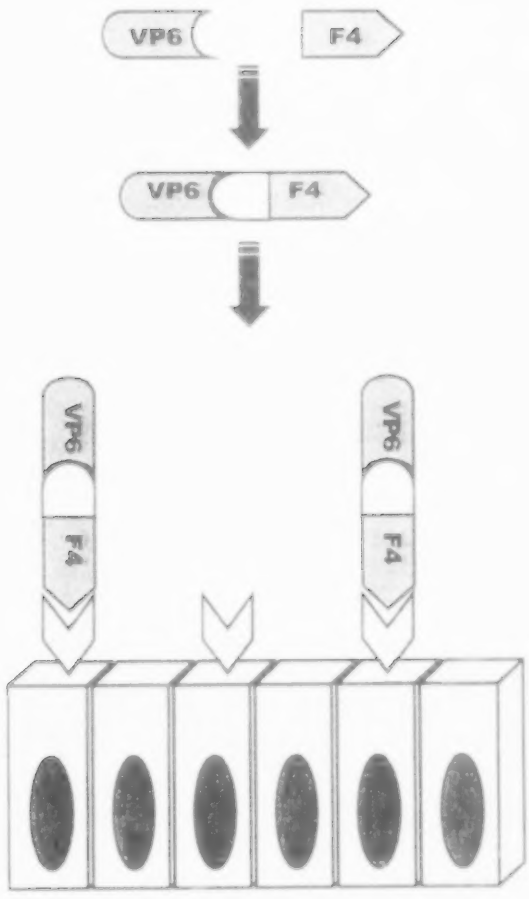


Fig. 9: Novel strategies for oral vaccines: Immunization against rotavirus. A fusion protein of F4 and rotavirus VP6 can be created with the goal of targeting VP6 to the intestinal lining via the F4 receptor (F4R), and thus improving the delivery of VP6 to immune cells.



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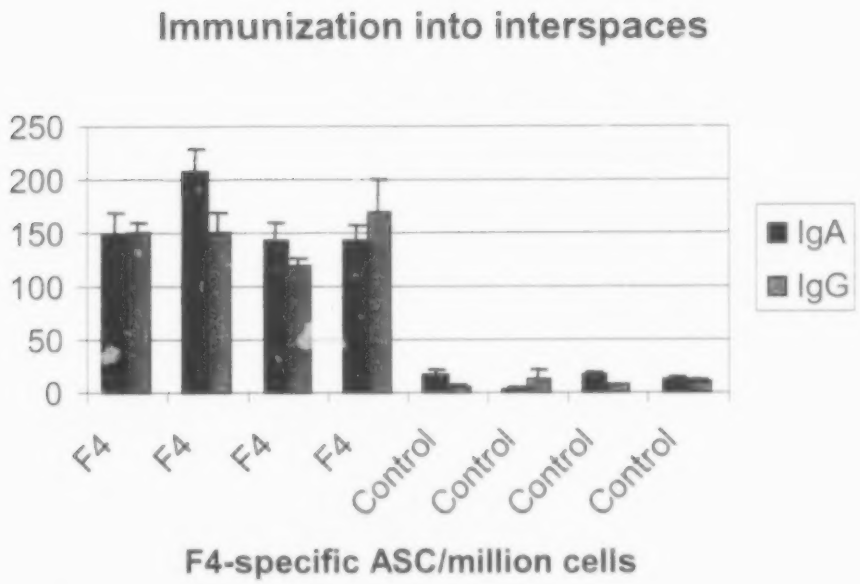

Fig. 10: Piglets were immunized with 500 µg F4 into four interspaces per piglet. Four interspaces were treated with saline as control. The number of F4-specific IgA- and IgG-antibody secreting cells was determined in a modified ELISPOT assay. Data for a F4R+ pig are shown.

Table 1: List of Experiments

Experiment	Purpose	# animals	Immunization/ Treatment	Immune response
I.	Challenge infection	10 10	Group I = ETEC *E.coli* Group II = PBS	8 / 10 symptoms 0/ 10 symptoms
II.	Challenge infection	9 6	Group I = ETEC F4 Group II = PBS	9 /9 symptoms, 6/ 9 died 0/ 6
III.	Local immune response against F4 (gut-loop)	4	- 250 µg purified F4 per loop	1 / 4
IV.	Local immune response against F4 (gut-loop)	4	- 500 µg F4 per loop (2 loops) - 500 µg OVA per loop (2 loops) - 10 µg CT per loop (2 loops)	4 / 4
V.	Dose response	6	- 50 µg F4 per loop (2 loops) - 250 µg F4 per loop (2 loops) - 500 µg F4 per loop (2 loops)	5 / 6
VI.	F4 plus CpG DNA	6	- 50 µg of F4 (3 loops) - 50 µg of F4 (3 loops) plus CpG (500 µg)	4/ 6
VII.	F4 plus pBD-1	7	- 50 µg of F4 (3 loops) - 50 µg of F4 (3 loops) plus pBD-1 (500 µg)	4/ 7